Subversion of Host Recognition and Defense Systems by *Francisella* spp.

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INTRODUCTION

Francisella tularensis was first identified as the causative agent of a fatal plague-like disease in a population of ground squirrels in Tulare County, CA, in 1911 ([147]). Originally called *Bacterium tularense*, it was later renamed *Francisella tularensis* in honor of Edward Francis, who spent his career extensively studying and characterizing the transmission and growth of this bacterium ([209]). Although it causes disease in squirrels, rabbits, and numerous other mammals, no animal has been identified as a reservoir. Instead, the reservoir may be freshwater or amoebae living therein. As there is no person-to-person spread, *F. tularensis* is acquired primarily by humans via arthropod vectors or zoonotic transmission, though it can also be transmitted by inhalation of aerosolized bacteria or ingestion of contaminated food or water ([2]). Inhalation of *F. tularensis* causes the most severe infections, and only 10 bacteria can lead to a potentially fatal disease. This high infectivity, along with its ease of aerosolization, have led to its history of weaponization ([209]).

Francisella species are endemic only in the Northern Hemisphere. *F. tularensis* subsp. *tularensis* (*F. tularensis*) is the most virulent etiologic agent of tularemia in humans and is the primary disease-causing *Francisella* species in North America. *Francisella tularensis* subsp. *holarctica* (*F. holarctica*) is responsible for the majority of reported cases of tularemia in Europe and Asia. The current vaccine is an attenuated live vaccine strain (LVS) derived from virulent *F. holarctica* by serial passage. LVS causes a very mild infection in humans but can cause a lethal infection in mice and is therefore commonly used as a model to study *Francisella* pathogenesis. The closely related *Francisella novicida* species rarely causes disease in humans, though some cases have been documented ([31], [125]). However, *F. novicida* is highly virulent in mice, has over 98% identity to *F. tularensis* at the DNA level ([188]), shares many of the same virulence genes ([43]), and is also used as a

model system to study *Francisella* virulence. Finally, *Francisella tularensis* subsp. *mediasiatica* is a species of intermediate virulence in humans and is found in Central Asia, while *Francisella philomiragia* and *Francisella noatunensis* can cause infections in aquatic organisms, including wild and farmed fish ([57]). Throughout this paper we will refer to "*Francisella*" when discussing general characteristics shared by numerous species and subspecies and will otherwise refer to specific species and subspecies by name. It is important to note that there are significant differences between highly virulent and less pathogenic strains in terms of the requirements of genes for pathogenesis, susceptibilities to host defenses, and the types of immune responses induced. Therefore, caution must be used when interpreting results from experiments using less pathogenic species and drawing conclusions about the characteristics of highly virulent species.

F. tularensis subspecies are the etiological agents of the disease tularemia, also known as rabbit fever. Tularemia is characterized by a 3- to 5-day incubation period ([209]) during which the bacteria replicate almost "silently" in macrophages and other types of host cells. The eventual release of bacteria from these cells may coincide with the presentation of flu-like symptoms. There are several manifestations of tularemia, each dependent on the route of acquisition ([159]). The most common form of tularemia is ulceroglandular disease, which can result from insect bites or from contact with infected animal tissues following mechanical damage to the skin. A cutaneous ulcer develops at the site of infection, and
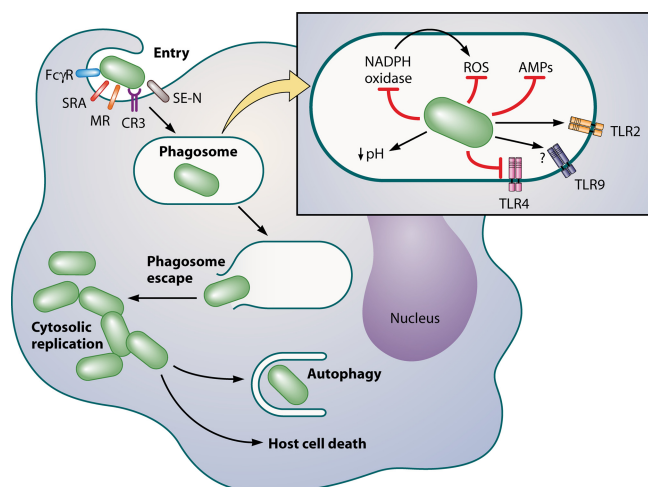
FIG 1 Stages of *Francisella* pathogenesis in the macrophage. *Francisella* can be detected by multiple macrophage receptors (see "Mechanisms of Entry and Fate of Intracellular *Francisella*" below) and is engulfed by a unique pseudopod loop mechanism. It then traffics to an early phagosome called the *Francisella*-containing phagosome (FCP). *Francisella* uses multiple mechanisms to evade host defenses in this harsh environment (inset). *Francisella* blocks the NADPH oxidase and also detoxifies reactive oxygen species (ROS). It can also resist the action of antimicrobial peptides (AMPs). *Francisella* does not signal through TLR4 but does activate TLR2 and may induce TLR9 signaling. *Francisella* then escapes the FCP to replicate within the cytosol. Subsequently, *Francisella* associates with autophagosomes, although the outcome of this interaction is unknown. *Francisella* can also induce host cell death.

bacteria drain to lymph nodes, subsequently causing a systemic infection. Less common forms of the disease include pneumonic, oculoglandular, and oropharyngeal tularemia. Streptomycin or doxycycline is indicated for treatment. Tularemia may be fatal; however, survivors gain robust immunity that has been found to last for up to 30 years (79).

Upon infection, *Francisella* initially comes into contact with extracellular defenses such as complement, antibody, and cationic antimicrobial peptides (28, 29, 51, 190). Binding of these components to bacteria directly or indirectly leads to lysis and killing (189). Therefore, *Francisella* uses multiple surface structures and outer membrane modifications (capsule, lipopolysaccharide [LPS] O antigen, modifications that increase surface charge, etc.) to resist these components and block killing. In addition, this prevents structural damage that would release proinflammatory bacterial components capable of initiating a strong immune response. *Francisella* also enters host cells as an efficient way of evading extracellular defenses.

After engulfment by phagocytic cells, including macrophages, *Francisella* is taken up into phagosomes that contain an array of toxic antimicrobials aimed at degrading the bacteria (Fig. 1). However, this pathogen has an equally diverse cache of defenses to counteract host antimicrobials. These once again prevent not only killing but also the release of proinflammatory bacterial components that could be recognized by host innate immune receptors (including Toll-like receptors [TLRs]) that stimulate inflammatory responses. Furthermore, similarly to entering host cells to avoid extracellular antimicrobials, *Francisella* escapes the phagosome to avoid phagosomal antimicrobials and, importantly, reach the cytosol, where it can replicate (Fig. 1). The cytosol is also, however, guarded by innate recognition and defense systems (in-

cluding the inflammasome) with which the bacteria must contend. In order to replicate in this host compartment, *Francisella* must also obtain the nutrients required to sustain its rapid cell division and actively counteract host defenses aimed at limiting nutrient availability.

In addition to subverting extracellular and intracellular defenses to facilitate replication, *Francisella* suppresses the activation of adaptive immune defenses that have the capability of destroying infected host cells. For example, CD8 T cells can directly lyse infected host cells, clearing these havens for bacterial replication. They and other cells also secrete the cytokine gamma interferon (IFN-γ), which can activate strong defenses in host cells, allowing them to resist *Francisella* replication. Therefore, *Francisella* uses several strategies to skew adaptive responses and block IFN-γ signaling to help preserve its replication niche in infected host cells (35, 119, 166, 189). Here we review the multitude of ways in which *Francisella* subverts host defenses at each of the stages of intracellular infection as well as its effects on adaptive immune responses.

SUBVERSION OF HOST DEFENSES

Following its transmission, *Francisella* must resist extracellular host defenses prior to entry into host cells. Furthermore, there is evidence that a potentially important extracellular phase exists after the initial stage of infection (87, 136, 239). Up to 75% of viable LVS and *F. tularensis* SchuS4 bacteria in the blood are present extracellularly at multiple time points following intranasal and intradermal murine infection (87). This finding was confirmed in studies with *F. novicida* in which ~80% of viable bacteria in the blood were present extracellularly in serum (239). Of important note, these extracellular *Francisella* organisms are still infectious, suggesting that this extracellular phase may play an important role in dissemination from the site of infection (239). These data highlight the fact that *Francisella* must subvert extracellular host defenses to initiate infection and likely to spread and cause disease at later stages as well.

Complement and Antibody

During extracellular phases of infection, *Francisella* must be able to subvert, resist, or evade killing by a plethora of antimicrobial defenses. One major extracellular defense is the complement system that is present and active in mammalian blood. The complement system consists of an intricately regulated cascade of extracellular signaling proteins that can be triggered by the recognition of a microbe, ultimately leading to its lysis by a pore-forming complex or promoting uptake and killing by phagocytes (190). Throughout the process of complement activation, host signaling molecules which act to promote inflammation and recruit phagocytes and T cells to the site of infection are formed. Furthermore, inhibition of complement activation is necessary to maintain the integrity and viability of the pathogen, as well as to prevent the release of immunostimulatory bacterial components (pathogen-associated molecular patterns [PAMPs]) that would activate an enhanced immune response.

Complement activation is a complex process and is described in detail in a number of reviews (88, 190). Briefly, this process is initiated by the binding of lectins or antibodies to bacteria, which subsequently promote the recruitment and activation of the complement factors C1 through C4. This leads to the generation of the C3 convertase, a central signaling molecule in the complement cascade. When C3 is cleaved to C3a (an anaphylatoxin) and C3b,
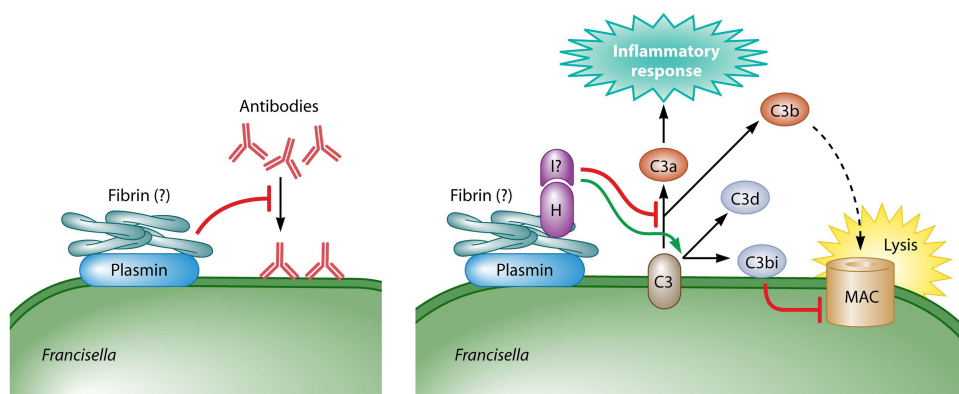
FIG 2 Complement Evasion by *Francisella*. Antibodies (Abs) can bind to the bacterial surface (left panel), leading to binding of complement component C3 (right panel), triggering activation of the complement cascade and generation of C3a, an inflammatory anaphylatoxin, and C3b. C3b ultimately leads to the formation of the membrane attack complex (MAC) and lysis of the bacterial cell. *Francisella* counteracts these defenses by binding host plasmin, which inhibits antibody binding (left panel) and thus complement activation. *Francisella* also binds host factor H, which inhibits C3 cleavage to C3a and C3b and instead acts with host factor I to create the complement-inhibitory molecules C3d and C3bi. This skews the complement cascade from promoting MAC formation and lysis to facilitating phagocytosis. Factor H binding may be promoted by fibrinogen, which is converted to fibrin and is known to bind plasmin, but this has yet to be demonstrated.

the canonical complement cascade continues, ultimately leading to bacterial lysis (Fig. 2). Briefly, lysis occurs when C3b, bound to the bacterial envelope, interacts with other complement factors to signal for the formation of the membrane attack complex (MAC). The MAC forms a pore in the bacterial envelope, leading to loss of membrane integrity and osmotic potential, in turn leading to lysis of the bacterium.

Although C3 binds to the bacteria, *Francisella* blocks complement activation by cleaving this protein into the smaller inhibitory fragments C3bi and C3d (Fig. 2) (28, 29, 51). Generation of these fragments prevents the ability of C3 to signal toward MAC formation. The exact mechanism of C3 inactivation on the *Francisella* surface is not yet known; however, host factor H readily binds the *Francisella* cell surface (29) and can inhibit formation of the C3 convertase (Fig. 2). Importantly, factor H can also serve as a cofactor for host factor I-mediated cleavage of C3b to C3bi and C3d. Despite the evidence that factor H binds *Francisella*, factor I (which is necessary for C3b cleavage) has not yet been shown to interact directly with the bacterial surface. Factor I may interact only transiently, or if it does not bind, a *Francisella*-encoded factor may be responsible for C3b cleavage to C3bi and C3d.

Generation of C3bi and C3d is not only responsible for inhibiting subsequent MAC formation but is also important in promoting opsonophagocytosis. By binding complement receptor 3 (CR3) on host phagocytes, C3bi and C3d allow *Francisella* to enter host cells, further promoting escape from extracellular antimicrobials such as complement, antibody, and antimicrobial peptides (20, 28) and facilitating entry of the bacteria into an intracellular replicative niche. It should be noted, however, that uptake mediated by C3bi/C3d and CR3 leads to limited *Francisella* replication compared to uptake by nonopsonic receptors (see "Mechanisms of Entry and Fate of Intracellular *Francisella*" below for a more detailed discussion). Therefore, *Francisella* encounters complement upon infection and can prevent complement-mediated lysis by converting C3 into C3bi and C3d. This routes *Francisella* to a nonoptimal replicative pathway within host cells, but it is nonetheless an effective way to subvert extracellular antimicrobials and promote intracellular replication.

Francisella's complement-inhibiting activity is highly dependent on the O-antigen oligosaccharide present on LPS on the surface of the bacterial cell (51, 192, 211). Mutants lacking O antigen, either by random selection for rough or gray variants or by targeting *wbt* locus genes necessary for O-antigen production, are significantly more sensitive to complement than their wild-type counterparts (51, 192, 211). Interestingly, the relative importance of O antigen in complement resistance differs between *F. novicida* and *F. tularensis*. *F. novicida wbt* mutants are more than 2,000-fold more sensitive to serum than wild-type bacteria, while *F. tularensis* mutants are only 4-fold more sensitive (221). This may be due to the structural differences in O-antigen tetrasaccharide oligomers between these species (221) and/or additional O-antigen-independent mechanisms of complement resistance in *F. tularensis* that have not yet been elucidated.

The sensitivity of *Francisella* O-antigen mutants to complement is due to increased binding of complement factors compared to that in the wild type and an inability to catalyze the cleavage of C3 to C3bi (51, 192, 211). This results in the binding of complement factors C5 to C9, which form the MAC and do not bind wild-type *Francisella*, ultimately causing lysis of the mutant bacteria and decreased survival in serum (29, 51, 192, 211). Therefore, the presence of the O antigen is vital not only for regulating the quantity of complement factors which bind to the *Francisella* envelope but also for subverting the ability of bound complement components to mediate lysis of the bacteria.

The importance of maintaining resistance to complement lysis is demonstrated by the fact that complement-sensitive mutants are also severely attenuated *in vivo*. *Francisella* strains with mutations in the *wbt* locus are attenuated during infection of both macrophages (130, 135, 221) and mice (129, 153, 204, 221, 232). Furthermore, mutants lacking genes necessary for O-antigen production that are outside the *wbt* locus are also attenuated for intracellular replication and *in vivo* virulence compared to the wild type (130).

Additionally, strains with mutations in genes predicted to be important for capsule production are also significantly attenuated *in vitro* and *in vivo* (throughout this paper, "*in vitro*" refers to
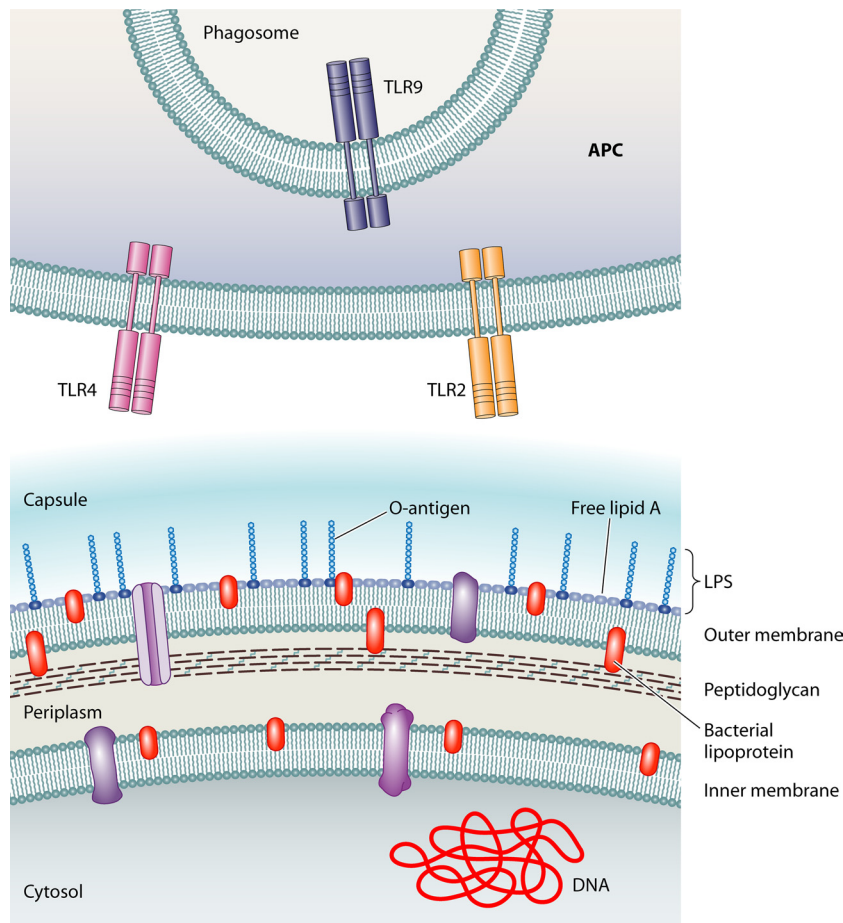
FIG 3 Shielding of inflammatory PAMPs in *Francisella*. *Francisella* modifies and limits the release of its inflammatory components (PAMPs) that can be recognized by pattern recognition receptors, such as TLRs, expressed by antigen-presenting cells (APC). *Francisella* bacterial lipoproteins (BLPs) and DNA (shown in red) are PAMPs with inflammatory activity, while the capsule, LPS, free lipid A, and membrane phospholipids do not elicit an inflammatory response. *Francisella* modifies its LPS and free lipid A such that these PAMPs do not activate host TLR4. It is unclear whether *Francisella* peptidoglycan (PGN) has inflammatory activity (represented in brown). Membrane proteins that are not BLPs and are not as a class considered PAMPs are shown in purple. The capsule and LPS O antigen provide resistance to antimicrobials such as complement and antimicrobial peptides, preventing damage to the bacterial membranes, which would result in the release of immunostimulatory BLPs and DNA. In addition to providing resistance to the damaging effects of antimicrobials, the capsule and O antigen may also serve as physical barriers to the release of PAMPs into the environment.

infection of host cells in culture, while "*in vivo*" refers to infection of mammalian hosts such as mice) (110, 130, 153, 232). This is important to note, since recent evidence has suggested that the O antigen and capsule of *Francisella* are structurally similar, with the capsule consisting of a polymer of O-antigen tetrasaccharide subunits (9) as well as a glycoprotein component termed a "capsule-like complex" (18). As such, some mutants deficient in LPS O-antigen production are also impaired in the production of capsule (9, 130), including specific *wbt* operon mutants (*wbtA*, *wbtC*, *wbtM*, and *wbtI*). *wbtK* and *lpxL* mutants lose the ability to synthesize LPS O antigen but maintain the polysaccharide capsule (9). Conversely, the *F. tularensis* genes *FTT_0673* and *FTT_0674* are necessary for capsule production but dispensable for O-antigen production (9). However, to the best of our knowledge, the ability of these mutants to resist complement has not been established. Testing the level of complement resistance in these mutants would allow the elucidation of which structure(s) is required. Thus, the current literature suggests that complement resistance in *Francisella* depends on O antigen, an O-antigen poly-

saccharide capsule, or both, as well as other potential factors that have yet to be elucidated.

Interestingly, the *Francisella* genome is known to contain an operon with similarity to the *Bacillus* species *capBCA* genes necessary for the production of a poly-D-glutamic acid capsule (71, 110, 153). However, to date, no poly-D-glutamic acid has been identified in *Francisella*. Although *Francisella capB* mutants are attenuated during murine infection, an LVS *capB* mutant did not exhibit increased sensitivity to serum (110, 213). Furthermore, it is not known whether CapB is required for production of the O-antigen capsule, the glycoprotein capsule-like complex, or a different bacterial envelope structure.

In addition to their sensitivity to complement, recent work has demonstrated that LPS O-antigen and capsule mutants are significantly more proinflammatory than wild-type cells (153, 171). An important, but perhaps overlooked, aspect of O-antigen and capsule function may be the ability of these large surface structures to mask and prevent the release of PAMPs on the *Francisella* surface (Fig. 3). In support of this hypothesis, LPS O-antigen mutants

release more DNA into the cytosol of host cells during infection, leading to an increase in host inflammation and proinflammatory cell death (discussed further in "Cytosolic Defenses" below) (171). However, it is unclear whether this release of bacterial DNA (a PAMP) is due to a general instability of the *Francisella* envelope or an increased sensitivity to serum components and/or other antimicrobials. Taken together, these data highlight the important role that polysaccharide surface structures play in preventing host recognition of *Francisella* and virulence.

In addition to the strategies described above, some bacteria use additional mechanisms to inhibit complement activation. For example, *Streptococcus pneumoniae* uses its surface M protein to bind host fibrinogen, a protein that can bind the complement inhibitor factor H, thus allowing fibrinogen-coated bacteria to inhibit complement activation (233). To our knowledge, no study to date has demonstrated that *Francisella* is capable of binding fibrinogen. However, recent work has shown that *Francisella* binds host plasminogen on its surface, which is in turn converted to plasmin (54), which can bind fibrinogen (137). Thus, although not yet directly demonstrated, this may be the mechanism used by *Francisella* to bind and sequester factor H, contributing to complement resistance (Fig. 2). Plasmin can also degrade soluble antibody, allowing *Francisella* to prevent antibody-mediated complement activation (59). Highlighting the potential importance of plasmin binding in pathogenesis, *F. tularensis* binds plasmin while the attenuated LVS strain does not, and LVS is therefore unable to evade this pathway. Together, *Francisella* utilizes LPS O antigen, its O-antigen polysaccharide capsule, and plasmin in order to subvert complement activation, promoting extracellular bacterial survival and preventing the release of proinflammatory PAMPs that would subsequently initiate a strong host immune response leading to clearance of the infection.

Antimicrobial Peptides

In addition to complement, the host produces a variety of other antimicrobials, most notably cationic antimicrobial peptides such as defensins and cathelicidins (42). Positively charged antimicrobial peptides, capable of disrupting the negatively charged bacterial membrane, are present extracellularly on mucosal surfaces as well as within macrophages and neutrophils. Upon infection, the bacteria first encounter either the epithelium of the lungs (after inhalation) or the skin (upon arthropod bites or contact with infected animal tissue). Subsequently, macrophages and neutrophils are among the first cell types the bacteria enter. Therefore, resistance to cationic antimicrobial peptides is likely a critical component of early pathogenesis.

Three defensin antimicrobial peptides (hBD-1, hBD-2, and hBD-3) are present in the human airway mucosa (42). The 50% effective concentration (EC_{50}) (the concentration that kills 50% of the bacteria) for hBD-1 and hBD-2 against *F. novicida* is higher than that against other bacteria such as *Pseudomonas*, *Staphylococcus*, and *Escherichia coli* (roughly 100-fold greater for hBD-1 and 10-fold greater for hBD-2) (108). Accordingly, hBD-1 and hBD-2 are ineffective at killing *F. novicida* (98), and at sites of infection, it is estimated that hBD-1 and hBD-2 concentrations are below the levels required for killing (198). It is likely that even though *in vitro* infection of lung epithelial cells with *F. novicida* induces the expression of hBD-1 and hBD-2 (10- and 40-fold, respectively), these levels are still too low to effectively kill the bacteria. hBD-3, however, is effective at killing *F. novicida* (in broth culture) (98),

but it is present at levels only about 2-fold higher than the EC_{50} (198), and infection does not further induce its expression (98). Therefore, it is likely that defensins play very little role in host defense against *Francisella*, due to its high resistance to hBD-1 and hBD-2 as well as its lack of induction of hBD-3 during infection.

Similar to the case for hBD-1 and hBD-2, *F. novicida* significantly induces the production of the cathelicidin LL-37 in lung epithelial cells *in vitro* (5). Furthermore, it is estimated that at sites of infection, LL-37 peptide concentrations average approximately 25 µg/ml (198), about 100-fold higher than the EC_{50} for *F. novicida* (5). Similar to its evasion of complement, *Francisella* must likely resist the action of LL-37 and presumably other host antimicrobials (such as hBD-1 and hBD-2) to prevent not only lysis and death but also the release of PAMPs that would subsequently trigger a proinflammatory response.

The literature to date has described at least two different mechanisms by which *Francisella* can either resist or evade the action of host antimicrobials: (i) by altering the charge of its surface and thus being able to use electrostatic interactions to repel cationic antimicrobial peptides and (ii) by encoding a number of efflux systems that are necessary for resistance to antimicrobials as well as for virulence *in vivo*.

Francisella modifies its lipid A, an outer membrane glycolipid structure that is a component of LPS but is also present in *Francisella* in a "free" form which lacks the canonical LPS core and O-antigen sugars (discussed in greater detail in "Toll-Like Receptors" below) (230). The bacteria remove the negatively charged phosphate groups from the 1 and 4′ positions of the lipid A portion of complete LPS (Fig. 4), which serves to increase the overall charge of the bacterial surface and repel cationic antimicrobials (94). Mutants that do not remove the 4′ phosphate are much more sensitive to cationic antimicrobial peptides and are highly attenuated *in vivo* (229). This attenuation is attributed to the increased susceptibility to cationic antimicrobials, which leads to membrane damage and the release of higher levels of PAMPs, resulting in a greater proinflammatory response, including increased neutrophil recruitment that results in clearance of the bacteria (210, 229).

Preventing interactions with cationic host antimicrobials is one of *Francisella*'s first lines of defense against the host immune response. However, increased surface charge does not prevent all antimicrobials from acting on the bacterial cell, particularly uncharged or negatively charged antimicrobials that could potentially be attracted more efficiently. *Francisella*, like many other bacteria, including *Neisseria* (95), *Pseudomonas* (179), and *Salmonella* (23), encodes at least one known multidrug efflux pump that allows resistance to a number of different antimicrobials and detergents, the AcrAB/TolC efflux pump. In addition, other predicted efflux pump proteins have been identified in screens for *Francisella* virulence genes, including *FTN_1066*, *FTN_1217*, *FTN_1277*, *FTN_1654*, and *FTN_1657* (130, 140, 232). While the exact functions of these predicted efflux proteins are unknown, they may play a role complementary to the known functions of the AcrAB/TolC efflux pump.

The AcrAB inner membrane efflux platform (30), coupled with the TolC outer membrane transporter (91, 178), forms a pump that facilitates the active efflux of toxic compounds and detergents from the bacteria, preventing their antimicrobial action. This multidrug efflux pump is important for *Francisella* resistance to β-lactams, tetracyclines, aminoglycosides, quinolones, detergents
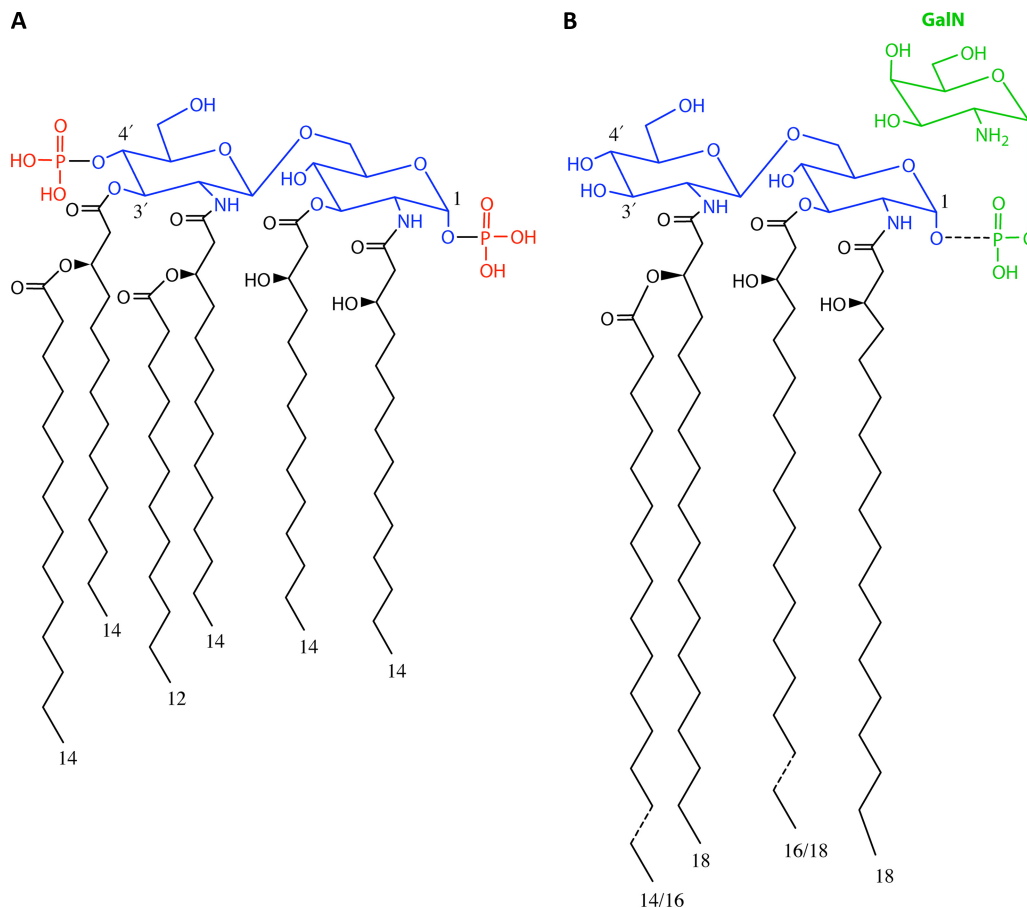
FIG 4 *E. coli* and *Francisella* lipid A structures. The structures of the *E. coli* lipid A moiety (A) and free lipid A from *Francisella* species (B) are shown. For both structures, the sugar backbone is highlighted in blue and acyl chains are represented in black, with numbers denoting length. Phosphate groups at the *E. coli* lipid A 4′ and 1 positions (absent from lipid A of full-length *Francisella* LPS) are highlighted in red. *Francisella* free lipid A contains the phosphate at the 1 position and is modified with galactosamine (GalN) (in green).

(notably bile salts), and antimicrobial dyes (30, 91). Highlighting the importance of this system in pathogenesis, mutants with mutations in pump components are severely attenuated *in vivo* (30, 91, 178). While the AcrAB/TolC system is known to provide resistance against host antimicrobial peptides in bacteria (95) and it is assumed that *Francisella* mutants would be more sensitive to host-derived antimicrobials during infection, to the best of our knowledge this has not been directly tested.

Interestingly, TolC mutants stimulate an increased proinflammatory response compared to that of wild-type *Francisella* (178). This may be due to increased sensitivity to antimicrobials *in vivo* leading to increased PAMP release or to a general membrane instability defect eventually leading to PAMP release. Furthermore, since TolC has been shown to be involved in the secretion of virulence factors in other bacteria such as *Pseudomonas* (100) and *E. coli* (227), it cannot be ruled out that it may play a similar role in *Francisella* pathogenesis, accounting for some of the virulence defects exhibited by mutants lacking this protein.

Another important aspect of antimicrobial peptide resistance in *Francisella* is the potential contribution of the O antigen and/or capsule. The *Klebsiella* capsule prevents interaction with antimicrobial peptides (39), and the O antigen of *Salmonella* has been shown to act similarly (212). However, to the best of our knowl-

edge, whether these systems contribute to *Francisella* resistance is not yet known. Together, the ability of *Francisella* to resist and evade the action of complement, antibody, and antimicrobial peptides within the host allows it not only to survive but also to maintain the integrity of its envelope, preventing the release of proinflammatory molecules that would induce a host response and promoting its virulence.

Mechanisms of Entry and Fate of Intracellular *Francisella*

As mentioned above, one mechanism used by bacteria to evade extracellular defenses is simply to enter host cells. Macrophages are sentinels of infection and are one of the first cell types to encounter *Francisella* (97). Upon initial contact with macrophages, *Francisella* binds to host receptors and is taken up via spacious, asymmetrical protrusions called pseudopod loops (52). Ultrastructural analyses have shown that pseudopod loops are formed during uptake of unopsonized and opsonized *Francisella* and are present in multiple cell types (52). This novel phenomenon differs from other described bacterial uptake mechanisms, including coiling phagocytosis seen at the surface of phagocytes during *Legionella pneumophila* infection and conventional phagocytosis observed during infection with multiple bacterial and viral species (102, 103, 199, 200).
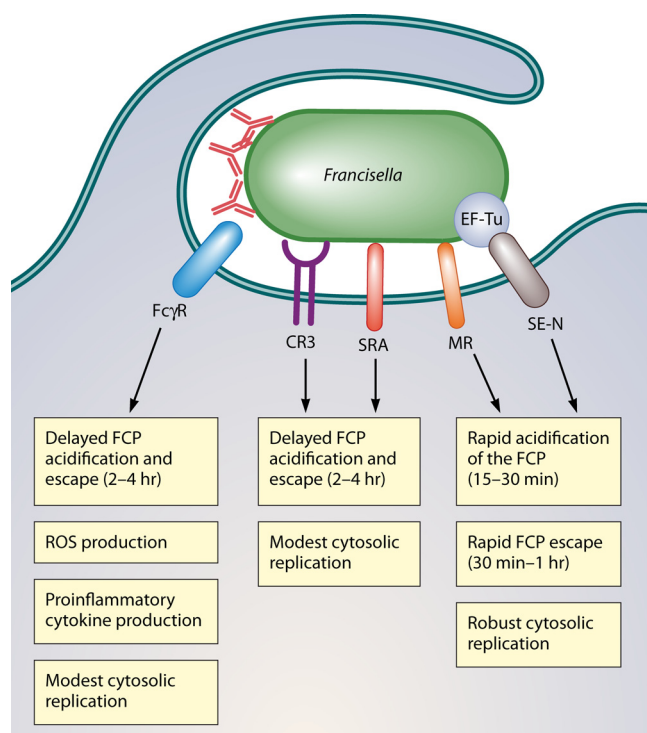
FIG 5 Intracellular fates of *Francisella* after uptake by different macrophage receptors. Antibody-opsonized *Francisella* is taken up via the FcγR, leading to increased ROS production and induction of proinflammatory cytokines, delayed FCP acidification and bacterial escape (2 to 4 h) from the phagosome, and only moderate levels of cytosolic replication. Uptake of serum (complement)-opsonized *Francisella* is mediated mainly by complement receptor 3 (CR3) and scavenger A receptors (SRA), which lead to slowed FCP acidification and phagosomal escape (2 to 4 h) and result in modest cytosolic replication. Lastly, uptake of unopsonized *Francisella* is mediated by the mannose receptor (MR) and surface-exposed nucleolin (SE-N), leading to rapid acidification (15 to 30 min) and escape from the FCP (30 min to 1 h) and robust cytosolic replication.

Furthermore, *Francisella* LVS can recruit host cell membrane cholesterol-rich lipid domains, or "lipid rafts," with caveolin-1 for successful entry into macrophages (217). Depletion of plasma membrane cholesterol or removal of glycosylphosphatidylinositol (GPI)-anchored proteins results in a severe decrease in *Francisella* uptake and subsequent intracellular replication (217). Interestingly, in nonphagocytic cells such as hepatocytes, LVS and *F. novicida* use cholesterol-rich lipid domains dependent on clathrin, not caveolin-1, for entry (123). Thus, *Francisella* uses multiple mechanisms for uptake into phagocytic and nonphagocytic cells.

The efficiency of *Francisella* uptake by macrophages depends in large part on whether the bacteria are opsonized, since serum- or antibody-opsonized bacteria are taken up by macrophages at 10-fold-higher levels than unopsonized bacteria (90). While this increased efficiency of uptake helps the bacteria evade extracellular defenses, it comes at a cost since the intracellular fates of opsonized and unopsonized bacteria are different (Fig. 5). Opsonized bacteria escape the phagosome with delayed kinetics and replicate modestly in the host cell cytosol (28, 90, 176, 202). In contrast, unopsonized *Francisella* escapes the phagosome rapidly and replicate robustly in the cytosol (17, 202). Therefore, the route of uptake has a profound impact on the outcome of infection, and

as discussed below, *Francisella* uses several strategies to promote entry mechanisms that favor its survival and replication.

Uptake of unopsonized *Francisella* depends largely on the mannose receptor (MR). When the MR is blocked with antibody or mannan, uptake of unopsonized *Francisella* decreases by more than 50% (17, 202). Unopsonized *Francisella* taken up by the MR escapes the phagosome rapidly and replicate to high numbers. In addition, the MR is known to induce relatively weak inflammatory responses compared to those induced by other phagocytic receptors (225). All of these outcomes favor the bacteria, and therefore, MR-mediated uptake makes host cells more permissive for *Francisella* replication, similar to what is observed during uptake of *Mycobacterium tuberculosis* (113).

In contrast, serum-opsonized bacteria are bound by complement, leading to uptake mediated in large part by complement receptor 3 (CR3). Ablation of CR3 (CR3$^{-/-}$ macrophages) results in at least a 50% decrease in uptake of serum-opsonized LVS (202). The scavenger A receptor (SRA), although traditionally known to play a role in uptake of unopsonized bacteria, contributes to the uptake of serum-opsonized *Francisella*, since SRA$^{-/-}$ macrophages exhibit a 20% reduction in the uptake of serum-opsonized LVS (176). Macrophage cell surface-exposed nucleolin has also been implicated in uptake of serum-opsonized *Francisella* by binding the bacterial membrane protein EF-Tu (19). Finally, MR-mediated uptake also plays a role in uptake of serum-opsonized *Francisella* (roughly 30% decreased uptake when blocked) (90). As mentioned above, this multireceptor uptake pathway predominated by CR3 results in delayed phagosomal escape and replication and is therefore nonoptimal for *Francisella*.

Opsonization with antibody, specifically IgG, leads to FcγR-mediated uptake of *Francisella*. FcγR$^{-/-}$ macrophages exhibit a 90% reduction in uptake of IgG-opsonized *Francisella*, although they have no defect in the uptake of unopsonized or serum-opsonized bacteria (90). FcγR-mediated uptake leads to increased activation of the NADPH oxidase, a phagosomal enzyme complex that produces toxic reactive oxygen species (ROS) and is discussed in greater detail below. This activation results in a more inhospitable environment for the bacteria in the phagosome, acting to delay phagosomal escape and severely limit subsequent bacterial replication. The efficacy of this host defense is revealed when ROS production is abrogated by genetic deletion of an NADPH oxidase component (using gp91$^{phox-/-}$ macrophages), since IgG-opsonized *Francisella* bacteria are rescued and can replicate to high levels (90).

Binding of plasmin by *Francisella* (mentioned in "Complement and Antibody" above) leads to degradation of soluble antibody at the bacterial surface (Fig. 2) (59), which limits uptake by the FcγR pathway and therefore subversion of this pathway, facilitating higher levels of intracellular replication. *Francisella* also subverts the CR3-mediated pathway using LPS O antigen and capsule, which inhibit binding of complement factors. It should be noted that when complement binding does occur, the bacteria alter the complement pathway to prevent MAC formation and lysis (Fig. 2). However, the alternate complement fragments generated actually promote phagocytosis via CR3. While uptake via this pathway is not optimal, it does still support bacterial replication and is a much better outcome for the bacteria than complement-mediated lysis. The most optimal entry pathway, however, is mediated via the MR. Therefore, *Francisella* uses the aforementioned subversion mechanisms to promote uptake by the more favorable

MR-mediated pathway, facilitating the highest levels of intracellular replication.

Phagosomal Acidification

After uptake by the macrophage, *Francisella* resides within the *Francisella*-containing phagosome (FCP) and subsequently escapes into the cytosol, where it can replicate (Fig. 1) (184). The brief time spent in the FCP is a dynamic step in infection during which *Francisella* must actively evade host antimicrobial defenses, including acidification of the FCP, reactive oxygen species, and antimicrobial peptides. Acidification of the phagosome is a defense mechanism that lowers the pH in this compartment, preventing many types of bacteria from efficiently replicating. Many intracellular pathogens must subvert this host defense mechanism by either blocking it, escaping the phagosome, or maintaining intracellular pH in spite of the lowered pH in the environment (184). It is interesting to note that acidification is actually required for the rapid phagosomal escape of the intracellular pathogen *Listeria monocytogenes* (27). Studies with *Francisella* have shown a brief acidification of the FCP that is dependent on the route of uptake, although there has been debate about whether this step is required for rapid escape and about its effect on the outcome of infection, as detailed below (49, 52, 53, 194).

Uptake of unopsonized *Francisella* leads to transient acidification of the FCP and rapid escape (49, 194). Santic et al. reported acidification of the phagosome at 15 to 30 min postinfection of human monocyte-derived macrophages (MDM) with unopsonized *F. novicida*, followed by escape at 30 min to 1 h postinfection (194). Interestingly, they showed that inhibition of the vacuolar proton ATPase (vATPase) pump by bafilomycin A (BFA) does not block escape but results in delayed escape (6 to 12 h). These results suggest that acidification is required for rapid escape from the FCP (194). Additionally, Chong et al. observed brief acidification of the FCP in macrophages infected with unopsonized *F. tularensis* (49). Similarly, BFA did not inhibit phagosomal escape or cytosolic replication but slowed infection kinetics (49). Together these data show that acidification of the FCP ensures rapid escape and robust replication of unopsonized *Francisella*; however, it is not required for these processes.

Concurrently, it has been reported that uptake of serum-opsonized *Francisella* results in acidification of a modest 20% to 30% of phagosomes containing serum-opsonized LVS and that it is not required for escape or replication (53). Additionally, the authors found that the maturing FCP does not acquire high levels of the acid hydrolase cathepsin D, which is a cellular marker of acidification, or the endosomal-lysosomal markers CD63, LAMP1, and LAMP2, which are cellular markers of phagosomal maturation (52, 53). In contrast to the previously mentioned work using unopsonized bacteria, BFA did not significantly delay infection kinetics in these experiments using serum-opsonized bacteria (53). These data demonstrate that acidification of the FCP is not required for escape and replication of unopsonized or serum-opsonized *Francisella* but is required for rapid escape of unopsonized *Francisella*. The fact that *Francisella* does not exhibit a significant survival or replication defect in the presence or absence of acidification demonstrates that this pathogen has devised ways to efficiently resist this host defense. This resistance is mediated in part by physically escaping the FCP, where acidification takes place (discussed in "*Francisella* Escape from the Phagosome" below). It

is also likely that *Francisella* has evolved mechanisms to maintain intracellular pH while in the acidified environment of the FCP.

Inhibition of Reactive Oxygen Species

In addition to subverting the potentially toxic effect of acidification of the FCP, *Francisella* must contend with reactive oxygen species (ROS) produced in this compartment. ROS are produced by the NADPH oxidase, a membrane-bound multicomponent enzyme system that converts molecular oxygen into toxic superoxide anions (161). In a resting phagocyte, NADPH oxidases are unassembled, with the gp91^{phox} and p22^{phox} (also called flavocytochrome b_{558}) components localizing to the plasma membrane and p47^{phox}, p40^{phox}, p67^{phox}, and Rac2 to the cytosol (145, 161). Upon phagocytosis of a microbe, the cytosolic subunits traffic to the phagosome and assemble with the membrane subunits to create the active NADPH oxidase that then produces ROS. Similar to the case for numerous extracellular and intracellular bacterial species, including *Helicobacter pylori* and *Salmonella* spp., multiple *Francisella* species block NADPH oxidase assembly in neutrophils and macrophages (4, 89, 145, 156, 203).

Francisella species use several approaches to inhibit ROS, including blocking initial assembly of NADPH oxidase components at the phagosomal membrane, blocking ROS production in complexes that have assembled, and detoxifying ROS that are generated. *F. novicida* requires four putative acid phosphatases (AcpA, AcpB, AcpC, and Hap) for inhibition of NADPH oxidase assembly (155, 186). AcpA colocalizes with the cytosolic NADPH oxidase component p47^{phox} during infection, and purified AcpA dephosphorylates p47^{phox} and p40^{phox} (155). Without phosphorylated membrane-bound components, the cytosolic NADPH oxidase components are not recruited and assembly cannot occur (14). These data suggest that AcpA interacts directly with NADPH oxidase components to block complex assembly.

While AcpA plays a role in limiting the oxidative burst, its relative contribution to virulence is unclear. Two studies found that AcpA is required for replication of *F. novicida* in human macrophage-like cells, indicating that blocking the NADPH oxidase is an important factor in facilitating replication (11, 155). In contrast, another study using *F. novicida* determined that AcpA did not play a role in replication in murine macrophages (22). In *F. tularensis*, deletion of *acpA*, or even of *acpA*, *acpB*, and *acpC* together, did not influence virulence in murine macrophages or human monocytes (48, 146). These conflicting data indicate that AcpA is not required for *Francisella* replication under all conditions tested. Its requirement may depend on the species or host cells used and the specific infection conditions.

In addition to blocking assembly, *F. tularensis* can also inhibit the generation of ROS when NADPH oxidase assembly is induced by exogenous stimuli (146). In spite of the formation of this complex, *F. tularensis* can inhibit the production of ROS. These data show that *F. tularensis* can block NADPH oxidase-dependent ROS production postassembly (146), although the mechanism by which this occurs has not yet been elucidated.

Although *Francisella* uses the mechanisms described above to significantly suppress activation of the NADPH oxidase, low levels of ROS are produced in the phagosome during infection (203). Like many pathogens, *Francisella* can directly detoxify ROS using proteins, including catalase (134, 151, 214) and superoxide dismutases (15, 16, 151), whose specific mechanisms of action are reviewed extensively elsewhere (135). In addition to these well-

known mechanisms of ROS resistance, we identified a previously uncharacterized protein, FTN_1133, that is required for virulence and resistance to organic hydroperoxides (135). FTN_1133 has sequence similarity to Ohr proteins, which are involved in resistance to organic hydroperoxides created during the interaction of ROS with lipids of the bacterial cell membrane (135). The *FTN_1133* mutant was attenuated for replication, although this could be restored in gp91$^{phox-/-}$ macrophages and mice (135). These data together indicate that *Francisella* has numerous overlapping mechanisms with which to subvert the NADPH oxidase and ROS, facilitating pathogenesis.

Francisella Escape from the Phagosome

Phagosomal escape is the last step in *Francisella*'s subversion of the phagocytic pathway, allowing it to escape the toxic phagosome and reach the cytosol, where it can replicate. The timing of escape has been a topic of debate and, as mentioned above, is largely dependent on the route of *Francisella* uptake by host cells. Unopsonized *Francisella* escapes the phagosome rapidly (within 1 h) whereas opsonized *Francisella* exhibits delayed escape (2 to 4 h) (92).

The exact mechanism of escape is not yet known; however, the proteins encoded in the *Francisella* pathogenicity island (FPI) are absolutely required. The FPI encodes a putative type VI secretion system (T6SS) that is essential for *Francisella* replication and pathogenesis (21; reviewed in reference 160). The requirement of a specialized secretion system for phagosomal escape would be in line with the situation in many intracellular bacteria, including *Shigella flexneri* (T3SS), *Listeria monocytogenes* (Sec pathway), and *Burkholderia* spp. (T3SS) (70, 168, 177, 193, 197). Briefly, the FPI proteins IglA and IglB share homology with proteins encoded in T6SS clusters in multiple bacterial species, are required for phagosomal escape, and may form the putative outer tube of the T6SS "needle" (64, 65). IglC has been proposed to form the inner tube (64), while IglI and VgrG are secreted and therefore may interact with host proteins during infection (21). In addition, numerous proteins that are not encoded in the FPI have also been implicated in phagosome escape and are reviewed elsewhere (11, 49).

To facilitate phagosomal escape, *Francisella* must subvert the action of host factors that have evolved to slow or block this process in order to control infection. Activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway by bacteria leads to production of proinflammatory cytokines, increased ROS production, and retention of bacteria in the phagosome (61, 104, 181). In accordance, activation of the PI3K/Akt pathway during *F. novicida* infection blocks phagosomal escape and cytosolic replication (181). Interestingly, *F. novicida* infection of macrophages activates the SH2 domain-containing inositol phosphatase (SHIP) by an unknown mechanism that is dependent on live bacteria (167). SHIP antagonizes activation of the PI3K/Akt pathway, leading to rapid escape from the phagosome and robust cytosolic replication (167). Additionally, the PI3K/Akt pathway can be activated by cell surface receptors, including FcγR (143) and TLRs (121). Therefore, *Francisella* mechanisms to evade these receptors (discussed in "Complement and Antibody" above and in "Toll-Like Receptors" below) may also function to block activation of the PI3K/Akt pathway and ensure rapid phagosomal escape.

Toll-Like Receptors

Upon contact with host cells, extracellular and intracellular microbes encounter host pathogen recognition receptors (PRRs) that are capable of detecting conserved microbial components (PAMPs) (109). These receptors can then trigger multiple pathways, including phagocytosis and inflammatory signaling (109) (115). Toll-like receptors (TLRs) are important PRRs that can recognize PAMPs outside the host cell and in the endosome/phagosome (115, 216). TLR signaling is mediated by TIR domain-containing adaptor proteins, including MyD88, TRIF, and TIRAP, that activate transcription factors such as NF-κB and IRF3 (115). These transcriptional regulators induce the expression of inflammatory cytokines and type I interferons, resulting in the activation of innate and adaptive immune cells (109, 115). Numerous TLRs recognize bacterial PAMPs, including TLR2, which senses bacterial lipoproteins (BLPs) and peptidoglycan (PGN) (12, 73), TLR4, which signals in response to LPS from Gram-negative bacteria, TLR5, which recognizes flagellin, and TLR9, which senses bacterial CpG DNA (115).

A central component of *Francisella*'s success as a pathogen is its ability to avoid recognition and subvert the host inflammatory response, particularly in the early stages of infection. Indeed, *Francisella* can likely evade or suppress inflammatory signaling by all of the aforementioned bacterium-sensing TLRs. For example, *Francisella* does not encode flagellin, and therefore TLR5 is not activated in response to infection (128). Though TLR recognition of *Francisella* DNA has been suggested (77), TLR9 (present in the membranes of endosomes and phagosomes) was shown to be unimportant for the host response to *Francisella* infection *in vivo* (56). This suggests that *Francisella* may subvert TLR9 activation, possibly by limiting the release of its DNA through resistance to damaging antimicrobial agents (see "Complement and Antibody" and "Antimicrobial Peptides" above) and/or by directly modulating TLR9 signaling. In support of the idea that *Francisella* maintains strong structural integrity to prevent DNA release, Peng et al. have shown that some hyperinflammatory *F. novicida* mutants exhibit increased bacteriolysis and DNA release during *in vitro* infection (171). Though *Francisella* rapidly escapes the phagosome (see "*Francisella* Escape from the Phagosome" above), it is intuitive that bacterial damage or killing and resulting DNA release during the time within this compartment would activate TLR9. It would be interesting to determine the extent of bacterial damage in the phagosome, whether phagosomal bacteria can trigger TLR9 signaling, and whether *Francisella* DNA is capable of acting as a TLR9 activator.

While TLR4 is considered a primary sensor of Gram-negative bacteria, *Francisella* LPS does not efficiently activate TLR4 compared to LPS from *E. coli* and other Gram-negative pathogens (180). Many Gram-negative bacteria that elicit robust TLR4 signaling synthesize a hexa-acylated lipid A portion of LPS with acyl chains of 12 to 14 carbons and phosphate groups at the 1 and 4′ positions (Fig. 4) (180). However, *Francisella* modifies or removes these important signaling structures. For example, *Francisella* lipid A acyl chains are two to six carbons longer than those in *E. coli* LPS (180). In addition, *Francisella* lipid A is tetra-acylated, as it lacks the canonical 3′ double acyl chain, and both the 1 and 4′ phosphate groups are absent (Fig. 4) (180). These modifications are critical for virulence, since an *F. novicida* mutant lacking the *lpxF* gene and producing a penta-acylated lipid A containing the 4′

phosphate group is rapidly cleared in a mouse infection model ([229]). This mutant exhibits hypersensitivity to the cationic antimicrobial peptide polymyxin B and elicits an increased local cytokine response and increased neutrophil recruitment *in vivo*. TLR4 is not involved in this response, suggesting that the attenuation is not due to an increase in the signaling capacity of the mutant LPS. Instead, it is likely that the observed attenuation is due to the mutant's increased sensitivity to antimicrobial peptides that lead to outer membrane damage and leakage of other bacterial PAMPs, such as BLPs and DNA, within the phagosome, where they could trigger TLR2- or TLR9-dependent inflammatory responses.

Unlike in other bacteria, between 70% and 90% of total *Francisella* lipid A exists as "free lipid A" that does not contain core polysaccharides or O antigen (Fig. 4) ([230], [243]). Similar to the case for the complete LPS, free lipid A is tetra-acylated with elongated acyl chains and lacks the 4′ phosphate group. However, instead of being removed as it is in complete LPS, the phosphate at the 1 position is present and modified with galactosamine ([230], [243]). This modification to free lipid A alone is critical for pathogenesis, since an *flmK* mutant that does not add the galactosamine to free lipid A is highly attenuated *in vivo* ([114]). To the best of our knowledge, free lipid A is unique to *Francisella* and could represent a novel mechanism of virulence that might be involved in strengthening the outer membrane. However, it is somewhat counterintuitive that *Francisella* would produce free lipid A without O antigen, since O antigen is so critical for virulence (see "Complement and Antibody" above). Further studies of the role of *Francisella*'s free lipid A during host interactions will elucidate its contribution to virulence.

Since *Francisella* LPS (and likely free lipid A) is a poor TLR4 activator, it is expected that TLR4 would not play a critical role in host defense against this pathogen. Most reports support this conclusion, although some disagree as to the magnitude of TLR4 signaling that *Francisella* LPS can elicit as well as whether TLR4 plays a minor role or no role in host defense. For example, studies have reported that, *in vitro*, LPS from *F. novicida* triggers a low level inflammatory response ([96], [116]) while LVS LPS does not, even at high concentrations (5 µg/ml) ([6], [116], [219]). However, other studies reported that LVS LPS can induce TLR4 signaling in both transiently transfected HEK293 cells and human monocytes, but only when added at high doses that are likely not biologically relevant (≥2.5 µg/ml) ([72]). The varied LPS signaling responses may be due to differences in the cell types used or slight structural variations, such as the 6′ glucose addition to *F. novicida* LPS or the 4′ or 6′ hexose modifications of the LVS LPS ([25], [94]). Overall, these data show that the LPS from the *Francisella* species tested is markedly less inflammatory than LPS from other Gram-negative organisms such as *E. coli*, *Salmonella*, or *Bordetella* ([6], [72], [96], [219]). This finding is in agreement with the general lack of TLR4 activation during *in vivo Francisella* infections, as discussed below.

Though early reports indicated that TLR4-deficient mice exhibit increased sensitivity to LVS infection ([8], [138]), the bulk of the current evidence suggests that TLR4 is not essential for the host response to infection. For example, TLR4-deficient mice are not more susceptible than wild-type mice to low-dose *F. tularensis* or LVS aerosol challenge ([46]), LVS low-dose intradermal (i.d.) infection ([47]), or intranasal inoculation with either LVS or *F. novicida* ([1]). However, the 50% lethal dose (LD_{50}) for LVS is 1 log lower in TLR4-deficient mice than in wild-type mice after high-dose i.d. infection (10^6 to 10^7 CFU) ([47]). Therefore, TLR4 may play a mi-

nor role in the host response to high-dose infections, though doubts remain about whether such a high inoculum is physiologically relevant. Even if TLR4 does play a minor role in host defense, it is not nearly as important as other innate defense proteins (tumor necrosis factor alpha [TNF-α], IFN-γ, or MyD88) that are critically required for resistance to infection as judged by the susceptibility to infection of mice lacking these proteins ([1], [126]). Overall, *Francisella* LPS is a poor TLR4 ligand, and TLR4 does not appear to play an important role in the host response to infection *in vitro* or *in vivo*.

TLR2 is the primary TLR involved in the inflammatory response to *Francisella* infection ([1]). Known *Francisella* TLR2 agonists include the uncharacterized lipoproteins LpnA/Tul4 and FTT_1103 ([86], [220]). To our knowledge, TLR2 recognition of *Francisella* PGN has not been reported, and the status of PGN as a TLR2 ligand is still under debate ([12], [73], [222]). Several reports have shown that TLR2 is essential for the early inflammatory response to *Francisella* infection in macrophages *in vitro*, as well as a critical component of the host response to *in vivo Francisella* infection as demonstrated by its requirement for control of pulmonary and intradermal infection ([1], [16], [55]).

However, while *Francisella* elicits TLR2-dependent signaling, it can also dampen this response. Specific *Francisella* genes that play a role in this suppression of the host inflammatory response have been identified. For example, a strain with a mutation in the FPI gene *iglC* not only failed to escape the phagosome or replicate in macrophages but also was unable to suppress TNF-α production and other inflammatory responses ([64], [131], [196], [219]). In addition, infection of macrophages with wild-type LVS blocked TLR2 and TLR4 activation in response to the addition of *E. coli* BLP and LPS, respectively, while the *iglC* mutant could not block this signaling ([219]). Further supporting the attenuation of TLR2 signaling by *Francisella*, infection with *F. tularensis* has been shown to reduce TLR2 expression ([38]). Mechanisms that the bacteria use to resist damage by antimicrobials, and therefore the release of BLPs, are also an indirect way of evading TLR2 signaling. It has been reported that high-molecular-weight (HMW) carbohydrates from "host-adapted" LVS and *F. tularensis* capsules impede TLR2-dependent cytokine production in murine macrophages ([240]), possibly by shielding the bacteria from antimicrobials. Taken together, these data indicate that *Francisella* is capable of subverting TLR2 signaling, while the host uses this pathway as a mechanism of innate defense.

Preliminary evidence suggests that highly virulent *F. tularensis* is able to suppress host recognition and inflammatory signaling even further. Melillo et al. recently reported that *F. tularensis* strain SchuS4 has a greater ability to prevent proinflammatory cytokine production than LVS during infection of murine macrophages ([150]). Indeed, 2 days after pulmonary infection, *F. tularensis* replicates to high numbers in the lungs of mice but does not induce inflammatory signaling and in fact triggers an increase in anti-inflammatory signaling ([7], [34], [35]). In addition, *F. tularensis* infection of dendritic cells (DC) suppresses the response of both infected and bystander cells to TLR agonists, due at least in part to a heat-stable bacterial component ([44]). Taken together, these findings suggest that *F. tularensis* is able to suppress the host inflammatory response to an even greater degree than other *Francisella* species, correlating with its heightened virulence. Further studies querying the role of TLR signaling in response to *F. tular-*

ensis infection could shed light on the mechanisms of inflammatory subversion utilized by this pathogen.

Finally, Collazo et al. demonstrated that mice lacking the adaptor MyD88, but not those lacking TLR2, TLR4, or TLR9, had greatly increased susceptibility to LVS infection (56). This indicates that multiple TLRs may play overlapping roles in resistance to infection, such that no one TLR knockout displays highly enhanced susceptibility (56). Alternatively, Medina et al. propose that an unknown MyD88-dependent PRR may be involved in the host response to LVS infection (148). Overall, *Francisella* species are able to largely evade recognition by TLRs as well as to suppress TLR2 signaling, significantly contributing to virulence.

Cytosolic Defenses

When *Francisella* reaches the cytosol, it has trafficked past TLRs and phagosomal defenses. It is nonetheless faced with a formidable challenge: replicate to high numbers without triggering an effective immune response. This is all the more challenging since the process of bacterial replication results in the release of PAMPs that can be recognized by cytosolic PRRs. Like the cell surface and the phagosome, the cytosol is equipped with numerous PRRs that recognize an array of bacterial products and elicit an immune response aimed at clearing the invaders. One large family of cytosolic PRRs is the Nod-like receptor (NLR) family, whose 22 members respond to a diverse set of PAMPs, including PGN (Nod1 and Nod2), flagellin (NLRC4, NAIP5, and NAIP6) (118), components of bacterial type III secretion systems (NLRC4), and damage induced by pore-forming toxins (NLRP3) (152). Nod1 and Nod2 are membrane-associated NLRs that detect muropeptide subunits of PGN and induce NF-κB activation and proinflammatory cytokine production (174). A role for Nods during *Francisella* infection has yet to be described, but these receptors may recognize and respond to PGN from this pathogen. Alternatively, *Francisella* may subvert Nods by modifying its PGN (similar to the case for *Listeria monocytogenes*, which *N*-deacetylates its PGN [33]) or by suppressing the Nod signaling pathway.

Other cytosolic PRRs, such as RIG-I and MDA-5, bind bacterial or viral nucleic acids and induce the production of type I interferons (IFNs) (157). The type I IFN family includes numerous IFN-α proteins, a single IFN-β protein, and other IFNs (66). These secreted cytokines have a well-established role in interfering with viral replication but can also be induced in response to bacterial infection (157). *F. novicida* induces type I IFN production independently of TLRs, Nod1/2, RIG-I, or MDA-5 (99). The cytosolic PRR that induces type I IFN production in response to *F. novicida* is currently unknown. However, the adaptor protein STING (stimulator of interferon genes) is required for type I IFN production during *F. novicida* infection and is speculated to act downstream of the yet-to-be-identified cytosolic PRR (112). STING is known to induce type I IFN production in response to cytosolic double-stranded DNA (dsDNA) derived from transfected plasmids, viruses, and some bacteria (107). Therefore, it is likely that *Francisella* DNA, which has been observed in the host cytosol by confocal microscopy (112), is the ligand that activates this cytosolic defense system.

It is not clear how *Francisella* DNA reaches the cytosol to trigger type I IFN, but bacterial escape from the phagosome is required for induction of this host response (99, 112). Perhaps damage incurred by *Francisella* in phagosomes allows for the release of DNA from ruptured phagosomes following escape. Interestingly,

an auxotrophic mutant of LVS that escapes the phagosome but cannot replicate in the cytosol failed to trigger cytosolic defense pathways (169), suggesting that bacterial replication might be required to increase the amount of DNA to a threshold level to which the host responds. In this context, mechanisms of maintaining structural integrity (LPS modifications, O antigen, and capsule; see "Complement and Antibody" and "Toll-Like Receptors" above) may prevent damage to the bacteria and the release of DNA in the cytosol and therefore would be hypothesized to promote subversion of these cytosolic defenses.

In addition to triggering the type I IFN pathway, cytosolic DNA released during *Francisella* infection can also be recognized by the PRR absent in melanoma 2 (AIM2) (82, 183), whose expression is upregulated by IFN-β (112). AIM2 is a member of the PYHIN (pyrin and HIN-200) family of proteins that binds dsDNA through a HIN-200 domain (81, 101, 187). AIM2 contributes to host defense by initiating the formation of a multiprotein complex called the inflammasome that is comprised of a PRR (from the NLR or PYHIN families), the scaffolding protein ASC, and the cysteine protease caspase-1 (144). Inflammasome activation causes infected cells to undergo an inflammatory form of programmed cell death called pyroptosis (84). This cell death may release bacteria into the extracellular environment, where they can easily be taken up by cells such as neutrophils that are not permissive for replication (210). Additionally, pyroptosis is accompanied by the release of the proinflammatory cytokines interleukin-1β (IL-1β) and IL-18 from dying cells, serving to recruit and activate other immune cells and further promote bacterial clearance. AIM2 inflammasome activation is essential for controlling *F. novicida* infection, since mice lacking components of this defense system succumb to infection much more rapidly than their wild-type counterparts (81, 141). The murine NLRC4 and NLRP3 inflammasomes do not play an obvious role in combating *F. novicida* during infection of macrophages or mice (141, 142). However, the NLRP3 inflammasome, along with the AIM2 inflammasome, is activated during *Francisella* infection of human epithelial cells and monocytes/macrophages (13). The mechanism underlying NLRP3 activation in human cells is not clear, since *Francisella* does not express known NLRP3 ligands.

Unlike the less virulent *F. novicida*, highly virulent *F. tularensis* fails to efficiently activate the inflammasome (234). Macrophages and dendritic cells infected with *F. tularensis* secrete very low levels of the inflammasome-dependent cytokine IL-18 *in vitro*, and there is very little caspase-1 activation induced in the spleens and livers of infected mice (234). As mentioned, microarray analysis of human monocytes infected with *F. tularensis* revealed that this pathogen downregulates the expression of several genes belonging to the TLR and type I IFN pathways (38, 60). TLR2 signaling is necessary for the expression of IL-1β and increases the rate of inflammasome activation during *F. novicida* infection (111), while type I IFN is essential for inflammasome activation (99). Therefore, hampering TLR2 and IFN signaling, two major host defense pathways that contribute to inflammasome activation, could lead to a lack of activation of this complex during *F. tularensis* infection. It is likely that highly virulent *F. tularensis* also has additional ways by which to limit inflammasome activation.

Several *Francisella* genes have been implicated in modulating inflammasome activation (82, 106, 224, 232). However, Peng et al. recently showed that these genes were not important for actively modulating the inflammasome (171). Instead, the increased induction of

macrophage death triggered by a panel of mutants lacking genes encoding membrane-associated proteins was due to increased bacteriolysis in the cytosol that allowed for the leakage of DNA and increased inflammasome activation. This study suggests that maintenance of membrane integrity is critical for *Francisella* to prevent the release of PAMPs and induction of the inflammasome. *F. tularensis* may also directly suppress inflammasome activation; however, genes important for direct suppression of the inflammasome complex by *F. tularensis* have not been identified. The presence of such genes in *F. tularensis* but not less virulent *Francisella* species could explain the divergence in activation of this complex.

During the latter stages of *F. tularensis* infection, infected cells undergo caspase-3-dependent programmed cell death or apoptosis. Unlike pyroptosis, this form of cell death is noninflammatory (120). Wickstrum et al. observed a significant increase in apoptosis in the livers and spleens of *F. tularensis*-infected mice between days 3 and 4 postinfection. This spike in apoptosis was preceded by an exponential increase in bacteria and antigen distribution in the infected organs (234). *F. tularensis* may direct host cells to undergo apoptosis instead of pyroptosis following cytosolic replication, facilitating dissemination to neighboring cells without triggering a strong inflammatory response. Additionally, phagocytosis of apoptotic bodies by activated macrophages impairs the ability of these cells to produce proinflammatory cytokines (80). In the context of an *F. tularensis* infection, these macrophages that have been rendered immunologically suppressed by taking up bacterium-containing apoptotic bodies could serve as reservoirs for further replication. Interestingly, it is not clear how *Francisella* egresses from infected cells and disseminates throughout the host. These impaired macrophages could also serve as Trojan horses, trafficking the bacteria systemically.

Another strategy commonly used by viruses and some pathogenic bacteria to exit host cells is to hijack the autophagic pathway (69). Autophagy is a process carried out by eukaryotic cells in which cytoplasmic material is engulfed into double-membrane-bound vacuoles called autophagosomes that subsequently fuse with lysosomes for degradation (127). This pathway was originally described as being important for maintaining cellular homeostasis, but it is now evident that it is also important for host defense. Following cytosolic replication, some *Francisella* organisms reside inside autophagosomes (45), although it is not clear whether this is a host-induced response to control infection or a pathogen-induced mechanism to promote virulence. *Francisella* autophagosomes contain major histocompatibility complex (MHC) class II (105), suggesting that the host might induce autophagy to degrade bacteria and present their antigens to activate the immune system and control bacterial replication. However, *F. tularensis* modulates the expression of *atg5*, *beclin 1*, and several other autophagy-related genes (60) during macrophage infection, supporting the notion that *Francisella* may act to subvert this host defense system. Furthermore, autophagy was recently shown to regulate the level of AIM2 inflammasome activation by targeting these complexes for lysosomal degradation (206). Therefore, hijacking the autophagic pathway could serve as a critical step in *F. tularensis* pathogenesis by hampering inflammasome-mediated bacterial clearance and providing this pathogen with a method by which to escape from host cells.

Replicating in the cytosol of host cells without inducing an inflammatory response is one of the most challenging yet critical immune subversion tactics employed by *F. tularensis* during infection. Although many of the virulence determinants that aid in evasion of cytosolic defenses are not currently known, it is likely that there are numerous genes necessary to subvert recognition by multiple PRRs, activation of cell death pathways, and killing via autophagy.

Nutritional Defenses

Although subverting host defenses in the cytosol is essential to facilitate intracellular replication and virulence, *Francisella* must also ensure that it can acquire sufficient nutrients in this host cell compartment to sustain its high growth rate. The host, however, has developed mechanisms that limit the availability of specific nutrients, providing a "nutritional" defense against pathogens. The fight over nutrients highlights another critical aspect of host-pathogen interactions.

Many *Francisella* genes annotated as being involved in metabolism, for both energy production and biosynthesis of macromolecules, have been identified in screens for genes important in intracellular replication and *in vivo* virulence (130, 135, 140, 214, 232). These screens identified genes required for growth during infection but not in rich media, and they therefore suggest that *Francisella* must utilize distinct metabolic pathways in order to successfully survive and replicate within the host, compared to those required for growth in broth. Mapping the genes identified in these screens onto predicted pathways has highlighted specific metabolic pathways critical to *Francisella* replication in the host, such as the biosynthesis of purines and pyrimidines as well as gluconeogenesis and glycolysis. The importance of these pathways in *Francisella* intracellular replication has recently been reviewed by Meibom and Charbit (149). We will focus on the mechanisms utilized by *Francisella* to directly access nutrients, such as iron and tryptophan, which are actively limited by the host during infection.

Iron, one of the most thoroughly studied nutrients in terms of host-pathogen interactions, is necessary for a variety of enzymatic functions in bacteria and is a vital component of various redox reactions that take place during growth. Within the host, nearly 99.9% of total iron is intracellular (37). Extracellular iron is highly insoluble, which thus represents a barrier to its uptake (36). Therefore, the little iron present outside host cells is associated with iron storage molecules such as heme, transferrin, and lactoferrin (36, 228). *Francisella* can overcome this first barrier to iron acquisition by entering host cells, where the iron content is substantially higher. However, even within host cells, iron is often sequestered by iron-containing redox enzymes and iron storage proteins such as ferritin and lactoferrin, making its acquisition by the bacteria challenging (36, 228).

Furthermore, the host has additional mechanisms to try to sequester iron away from pathogens as it responds to infection. Past work has demonstrated that when macrophages sense LPS or are stimulated with IFN-γ, the host cells respond by decreasing expression of the transferrin receptor (TfR) that shuttles iron-bound transferrin into the cell (158). These signs of infection also signal the host to increase production of ferritin, which binds and sequesters free iron within host cells (185). Together, these host strategies decrease the availability of free iron both extracellularly and intracellularly.

Due in part to these mechanisms, serum iron concentrations significantly decrease in otherwise healthy human volunteers during *F. tularensis* SchuS4 inhalational infection (170), as occurs during other microbial infections (231). This can be correlated
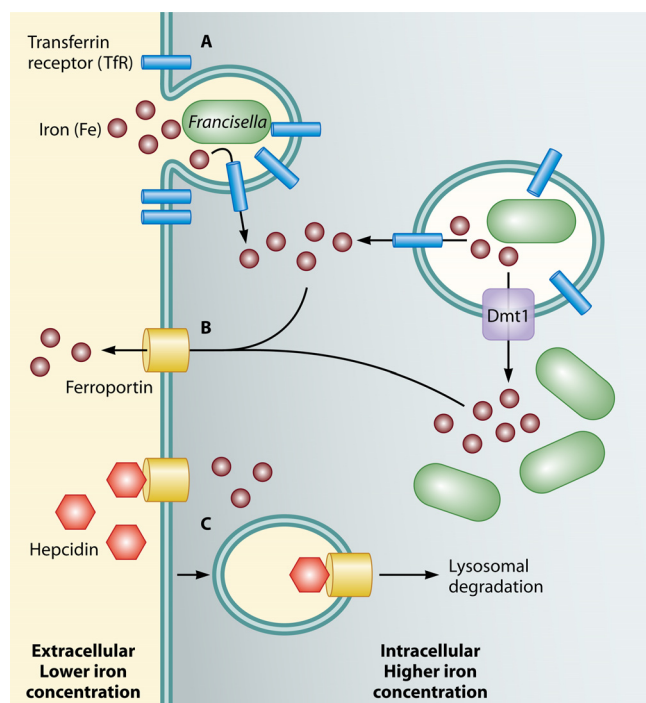
FIG 6 Competition for iron during *Francisella* infection. (A) Upon infection of macrophages, *Francisella* induces the upregulation and relocalization of the transferrin receptor (TfR) to the forming phagosome, facilitating an influx of iron into the host cell. (B) The host counteracts this by upregulating Dmt1 and ferroportin, which export iron from the phagosome to the cytosol and from the cytosol to the extracellular space, respectively. (C) *Francisella*, however, counters this by inducing host hepcidin, which binds ferroportin and causes it to be degraded. In total, this leads to an increase of intracellular iron available in the cytosol for *Francisella* replication.

with the fact that instead of the decrease in TfR expression typically observed in response to infection, TfR is significantly upregulated during LVS infection (165) and even colocalizes with the early *Francisella*-containing phagosome (FCP) (165). Interestingly, TfR localization does not occur with killed bacteria, indicating that LVS actively alters host TfR localization through an unknown mechanism (165). Increases in TfR would lead to increased intracellular pools of iron, while decreasing serum iron levels. Also, unlike stimulation with IFN-γ and/or LPS, LVS infection does not induce expression of ferritin (165). Therefore, LVS may also actively prevent the host from sequestering the intracellular iron brought in by TfR, allowing the bacteria to utilize the increased iron pool. Together, these changes in host expression of TfR and ferritin, as well as TfR localization to the FCP, cause a significant increase in usable intracellular iron that can enhance *Francisella* replication (Fig. 6).

Further highlighting the tug-of-war between host and pathogen, the host counters the influx of iron due to increased TfR expression during LVS infection by increasing expression of Dmt1, a phagosomal membrane protein which transports iron from phagosomes into the cytosol, as well as ferroportin, which shuttles iron from the cytosol out of the cell (Fig. 6) (165). The combined action of these proteins would limit the amount of intracellular iron available to replicating *Francisella* in the cytosol. Countering this restriction, LVS infection induces the production of hepcidin, an antimicrobial peptide secreted by hepatic cells

(165). Hepcidin binds to ferroportin on the host cell surface, causing it to become internalized and subsequently be degraded (162). Thus, hepcidin induction and subsequent inhibition of efflux of cytosolic iron appear to be one strategy employed by LVS to maintain intracellular iron pools at levels permissive for replication. However, whether LVS actively induces hepcidin production to specifically inhibit ferroportin or whether hepcidin's antiferroportin action is an unintentional consequence to the host and secondary to its antimicrobial activity is not known. The exact mechanisms by which *Francisella* promotes the expression of hepcidin and TfR, while simultaneously preventing ferritin expression, are unknown.

Further contributing to its ability to capture and acquire iron, *Francisella* utilizes a rhizoferrin-like siderophore (215). Siderophores are small molecules that selectively bind iron at a significantly higher affinity than host iron-sequestering compounds, creating a biochemical flux in favor of the siderophore-producing organism (154). This allows *Francisella* to outcompete the host for available iron. The *Francisella* locus necessary for production and utilization of its siderophore is *fslABCDE* (also termed *figABCDE*) (117, 215). *fslABCD* are directly involved in the production and/or export of the *Francisella* siderophore (117, 215). *fslE*, on the other hand, is not necessary for production or export, but instead is required for the utilization of the siderophore, through either siderophore binding or import (117, 182). *Francisella* lacks genes with significant similarity to the known (and typically highly conserved) siderophore receptor and importer genes *tonB*, *exbB*, and *exbD* (122). It is therefore hypothesized that *fslE*, or one of the two *fslE* orthologs present in the genome, may act as a novel siderophore importer (182). The two *fslE*-like genes, termed *fupAB* (encoded in LVS by a single protein fusion of these two paralogs, named *fupA/B*) (132, 205), are important for siderophore uptake, particularly in LVS compared to *F. tularensis*, which relies more heavily on *fslE* for siderophore import (132, 182, 205).

In the context of an infection, siderophore production and iron acquisition proteins are a vital component of *Francisella*'s ability to maintain iron homeostasis and ultimately survive and replicate in the host. *fslABC* and *feoB*, encoding a ferrous iron importer utilized by other bacteria to directly import free iron (41, 122, 215), have each been identified in *in vivo* virulence screens (214, 232). *fslABC* have also been shown to be necessary for replication in macrophages (135). Furthermore, *fupAB* are required for full virulence in mice during LVS (205) and *F. tularensis* (223) infection and for LVS replication in macrophages (140, 205). Interestingly, while unable to produce siderophores, an *fslA* mutant of *F. tularensis* SchuS4 is not attenuated during infection of mice, suggesting that other iron acquisition pathways, such as the *feoB* importer or the *fupAB* system, may be playing a parallel role. On the other hand, while not identified in previous screens for virulence factors, we have identified *fslE* in a negative selection screen in *F. tularensis* for genes necessary for survival in mice (D. S. Weiss et al., unpublished observation).

Although iron acquisition may play an important role in pathogenesis, recent work has demonstrated that *Francisella* must also limit its total intracellular iron concentration (133). Increased intracellular iron concentrations can lead to increased susceptibility to oxygen radical damage within the bacteria. In fact, more virulent strains, such as *F. tularensis* SchuS4, have lower levels of intracellular iron than the less virulent LVS, which correlates with increased resistance to oxygen radical-mediated stresses (133). It is important to note that these studies were performed using bacteria grown on agar plates. It would

be interesting to measure the iron content of each strain during infection to see if the differences mentioned above are maintained. Overall, these data suggest that *Francisella* maintains homeostasis of intracellular iron, importing enough to promote survival while preventing excessively high and toxic concentrations.

Although it is the most thoroughly studied, iron is not the only nutrient whose availability is actively limited by the host. The intracellular concentration of the amino acid tryptophan is significantly decreased during infection (218) due to an increase in the expression of indoleamine 2,3-dioxygenase (IDO), which initiates the tryptophan catabolic pathway by converting tryptophan to kynurenine (218). IDO is expressed in nearly every cell type, excluding hepatic cells, and is particularly highly expressed in the lungs and placenta (218). Infection by *Chlamydia* (26) or *Streptococcus* (172), as well as stimulation with IFN-γ (238) or LPS (237), induces IDO expression and results in decreased tryptophan levels. This is an important defensive mechanism, as it creates an innate nutritional barrier that limits the ability of microbes to replicate.

Upon intranasal infection with *Francisella*, IDO is significantly upregulated in the lung (172). *Francisella* is capable of overcoming this host defense by maintaining the ability to synthesize tryptophan *de novo*, although this process is very energy intensive (241). The importance of tryptophan prototrophy is exemplified by the fact that strains with mutations in *trpB*, which is necessary for the final step of tryptophan biosynthesis, are significantly attenuated during intranasal infection (50, 172) and that this attenuation can be overcome in IDO$^{-/-}$ mice (172). *trpB* mutants are capable of limited intracellular replication in macrophages and show decreased survival in IFN-γ-stimulated macrophages compared to wild-type *Francisella* (50). As IDO expression is induced by IFN-γ, the attenuation of the *trpB* mutant is also rescued upon infection of IFN-γ receptor (IFNGR)-deficient mice (50). These data indicate that tryptophan prototrophy is a necessary attribute of *Francisella* pathogenesis, allowing the bacterium to overcome an aspect of the nutritional barrier induced by IFN-γ and IDO upon infection of the lung.

While it can produce tryptophan, *Francisella* is auxotrophic for 13 amino acids which it cannot synthesize *de novo*, likely because it does not encode some or all of the enzymes required for their biosynthetic pathways (122, 149, 188). Therefore, it is intuitive that being able to acquire these essential amino acids within the host is a necessary component of *Francisella* pathogenesis. *Francisella* is predicted to lack all of the biosynthetic enzymes for arginine, histidine, lysine, methionine, and tyrosine (122). Interestingly, a putative methionine uptake transporter (*FTN_1107/FTT1125*) (140, 214), lysine importer (*FTN_0296/FTT1633c*) (232), and tyrosine permease (*FTN_1711/FTT1732c*) (11) have all been identified in virulence screens, suggesting that they are necessary for pathogenesis. Furthermore, amino acid importers with no predicted substrate (*FTN_0097/FTT1688* and *FTN_0848/FTT0968c*) have been identified as well (130, 135, 140, 214, 232). This suggests that *Francisella* may have developed strategies to scavenge from the host those amino acids which it cannot synthesize *de novo*.

Other than tryptophan restriction, to the best of our knowledge, we do not know of another example of active host depletion of an amino acid that is important during *Francisella* infection. While numerous biosynthetic and metabolic pathways have been lost during the reductive evolution of the more recently evolving *F. tularensis* and *F. holarctica* subspecies, *Francisella* species are capable of *de novo* biosynthesis of alanine, glutamate, glutamine, asparagine, glycine, phenylalanine, and tryptophan (122, 149, 188).

In fact, pathways for the synthesis of glutamate, glycine, and phenylalanine are redundantly represented. This suggests that the ability to synthesize these amino acids is essential for pathogenesis and could potentially counter active host restriction mechanisms, similar to the way *Francisella* counters tryptophan depletion as described above. In further support of this idea, asparagine synthase (*FTN_1421/FTT1456c*) which is necessary for asparagine prototrophy (130, 135, 232), and a number of enzymes utilized for the creation of aromatic amino acid precursors have been identified in virulence screens (149).

Another well-known example of *Francisella* auxotrophy is the requirement for cysteine, which serves as a necessary source of sulfate. This requirement is also necessary in broth and thus does not necessarily represent an active host defense process to deplete cysteine. However, in the host, cysteine is present within glutathione (GSH). The ability to utilize GSH is thus a required component of *Francisella* intracellular growth. Indeed, *F. tularensis* mutants lacking a transpeptidase (*ggt*) necessary for cleavage of GSH are severely attenuated during infection of macrophages and mice (3).

The requirement for other biosynthetic pathways during host infection, such as purine and pyrimidine biosynthesis, is well established for *Francisella* and other bacterial pathogens (10, 149). Furthermore, the importance of central metabolism (e.g., glycolysis) has also been shown to play a role during infection (149). Additionally, there is evidence that nutritional status may play a role in allowing *Francisella* to sense the host phagosome compared to being in the host cytosol (40) and to induce expression of virulence genes, including those required for phagosomal escape (40, 67, 191). Mutants that are unable to recognize particular metabolites, such as polyamines present in the host cytosol, are significantly attenuated and unable to alter temporal expression of virulence factors (191). Taken together, these data highlight the importance in the outcome of infection of host and pathogen proteins involved in this nutritional tug-of-war.

Modulation of Adaptive Immune Responses

We have focused a great deal of attention on the ways in which *Francisella* modulates the immune responsiveness of the cells that it directly infects. We now discuss the effect that these interactions have on the ability of other immune cells to fight infection and the development of an effective adaptive immune response. As noted previously, pulmonary infection caused by *F. tularensis* in humans is characterized by an initial delay in inflammatory responses during the first 72 h (58). Although it is not entirely clear how *F. tularensis* evades early immune responses, modulating the activity of innate immune cells that contribute to early cell-mediated immunity likely plays a significant role. Antigen-presenting cells (APCs), such as macrophages and dendritic cells, play an important role in early innate defense against *Francisella* infection. The ability of these cells to present microbial antigens and activate T cells enables them to serve as a bridge between the innate and adaptive immune systems. During the earliest stages of infection, *Francisella* resides primarily in macrophages (97). Therefore, it is not surprising that this pathogen uses multiple subversion mechanisms (e.g., facilitating cell entry via nonactivating macrophage receptors, escaping phagosomal killing, and modulating cytosolic defense pathways) to systematically disarm macrophage defenses. However, when activated by the proinflammatory cytokine IFN-γ, these phagocytes are capable of overcoming the modula-

tory effects exerted by *Francisella*, preventing its replication in the cytosol (68, 126). Primarily produced by NK cells and dendritic cells during infection, IFN-γ regulates over 200 genes, many of which are involved in host defense pathways important for enhancing nitric oxide production, inducing autophagy, and increasing major histocompatibility complex (MHC) class I and II antigen presentation (32, 226). Activation of these pathways could collectively enhance *Francisella* clearance and promote host survival. Moreover, a lack of IFN-γ *in vivo*, through either genetic disruption (76) or anticytokine treatment (126), increased the susceptibility of mice to *Francisella* infection, indicating that this cytokine is an important mediator of host defense *in vivo*.

The exact mechanism of IFN-γ-mediated clearance of *Francisella* from activated macrophages is unclear. IFN-γ can hinder *F. novicida* and LVS escape from the phagosomes of human monocyte-derived macrophages and murine peritoneal exudate cells, respectively (131, 195). This prolonged retention of *Francisella* in the phagosome would expose bacteria to phagosomal defenses such as the NADPH oxidase, nitric oxide, and lysosomal enzymes if phagolysosomal fusion occurred (201), all of which are upregulated by IFN-γ, leading to bacterial clearance. In contrast, escape of highly virulent *F. tularensis* from the phagosome is not affected by IFN-γ activation of macrophages, although its cytosolic replication is nonetheless restricted (74). Therefore, IFN-γ-mediated activation of macrophages leads to both phagosomal and cytosolic defenses that control the virulence of multiple *Francisella* species.

Since IFN-γ has such a pleiotropic effect on macrophage activation, any mechanism of suppressing the action of this cytokine would likely have broad effects on promoting *Francisella* pathogenesis. *Francisella* directly impairs the ability of macrophages to respond to IFN-γ stimulation during infection by downregulating the expression of the alpha subunit of the IFN-γ receptor (IFNGR), which is critical for signaling (189). Nallaparaju et al. found that in the event that signaling through the IFNGR does occur, *Francisella* uses FopC to indirectly block the phosphorylation and subsequent activation of Stat1 (a transcriptional regulator that is required for IFNGR signaling) (159a, 166). The IFN-γ signaling pathway is further decommissioned during infection as the bacteria induce an increase in the expression of SOCS3, a protein that negatively regulates this pathway (38). Activation of the TLR signaling pathway enhances IFN-γ-dependent cytokine production in macrophages (242). Therefore, suppression of TLR signaling by mechanisms discussed earlier in this review (prevention of PAMP release, modification of LPS/lipid A structure to prevent TLR4 activation, and downregulation of TLR expression) could also dampen activation of the IFN-γ pathway.

In addition to suppressing IFN-γ signaling, *F. tularensis* induces macrophages to produce the immunomodulatory lipid prostaglandin E$_2$ (PGE$_2$) (236). PGE$_2$ inhibits T cells from producing IFN-γ, favoring bacterial replication (Fig. 7) (175). PGE$_2$ also induces the expression of a >10-kDa protease-resistant host factor that promotes ubiquitin-dependent lysosomal degradation of MHC class II molecules, resulting in a nearly complete absence of MHC class II on the surface of macrophages (235). Therefore, by inducing PGE$_2$, *Francisella* inhibits bacterial killing mediated by IFN-γ and also dampens the induction of adaptive immune responses by limiting antigen presentation through MHC class II.

Dendritic cells play a critical role in directing cell-mediated immunity by producing cytokines that control the way in which T cells are activated and the immune responses that they subse-

quently promote. In accordance with this pathogen's ability to dampen cell-mediated immune responses, *F. tularensis* suppresses proinflammatory cytokine production from these cells (35). Among these cytokines is IL-12, which is important for the development of IFN-γ-producing T cells (75). Surprisingly, inhibition of IL-12 by *F. tularensis* is mediated by IFN-β production (Fig. 7) (24). As described previously in this review, type I IFNs contribute to inflammasome activation by *Francisella*. However, there was no correlation between IFN-β production and inflammasome activation during *F. tularensis* infection. Considering that *F. tularensis* dampens the expression of genes in the type I IFN pathway, it is possible that this pathogen suppresses the expression of type I IFNs to a level that is below the threshold for inflammasome activation but adequate for IL-12 suppression.

F. tularensis also redirects macrophages to become alternatively activated (207). Alternatively activated macrophages exhibit a skewed inflammatory profile that is less antimicrobial, with decreased levels of nitric oxide production upon IFN-γ stimulation and an impaired ability to kill bacteria (78, 93). Additionally, *F. tularensis* directs dendritic cells to produce the anti-inflammatory cytokines transforming growth factor β (TGF-β) and IL-10 (35, 236). This greatly contributes to the absence of an inflammatory response early in infection because IL-10 inhibits macrophage proliferation and proinflammatory cytokine production (164). In addition, it can lead to reduced MHC class II expression, which would suppress adaptive immune responses (139).

IL-10 or TGF-β can direct dendritic cells to become tolerogenic, inhibiting their ability to activate T cells that induce an inflammatory response (Fig. 7) (139). These cytokines also promote the development of regulatory T cells that can suppress the inflammatory activity of other T cells (85). In fact, pulmonary regulatory T cells develop in the lungs during *F. tularensis* infection, and this development correlates with an increase in bacterial burden (173). Though it is currently unclear how *F. tularensis* redirects APCs toward an anti-inflammatory response, inducing immune tolerance through the induction of IL-10 or TGF-β is a survival strategy used by pathogens such as *Yersinia pestis*, *Coxiella burnetii*, and *Chlamydia pneumoniae* (63). In the case of *Yersinia pestis*, IL-10 production is induced by the type III secretion system effector protein LcrV (124, 208). Further characterization of *Francisella* secretion systems and effector proteins may shed light on similar mechanisms of immune modulation.

Cell-mediated immunity is generally accepted as being essential for controlling intracellular bacterial infection. In accordance with this dogma, antibodies have been shown to provide little protection against *Francisella* infection, but antibody-independent B cell responses are important for early protection (62). However, *Francisella*, like some other intracellular bacterial pathogens (83), is also capable of infecting and replicating inside B cells (119), although it is not known whether it can modulate B cell activity in order to evade immune detection. *Salmonella enterica* serovar Typhimurium has been shown to induce MyD88-dependent IL-10 production in splenic B cells during systemic infection, promoting its survival *in vivo* (163). As discussed above, IL-10 induces a tolerogenic state in immune cells, and it is possible that *Francisella*-induced IL-10 production in APCs could also alter the function of B cells during infection.

Francisella uses multiple strategies to modulate APCs in order to subvert subsequent activation of the adaptive immune system. This modulation not only contributes to the failure in immune
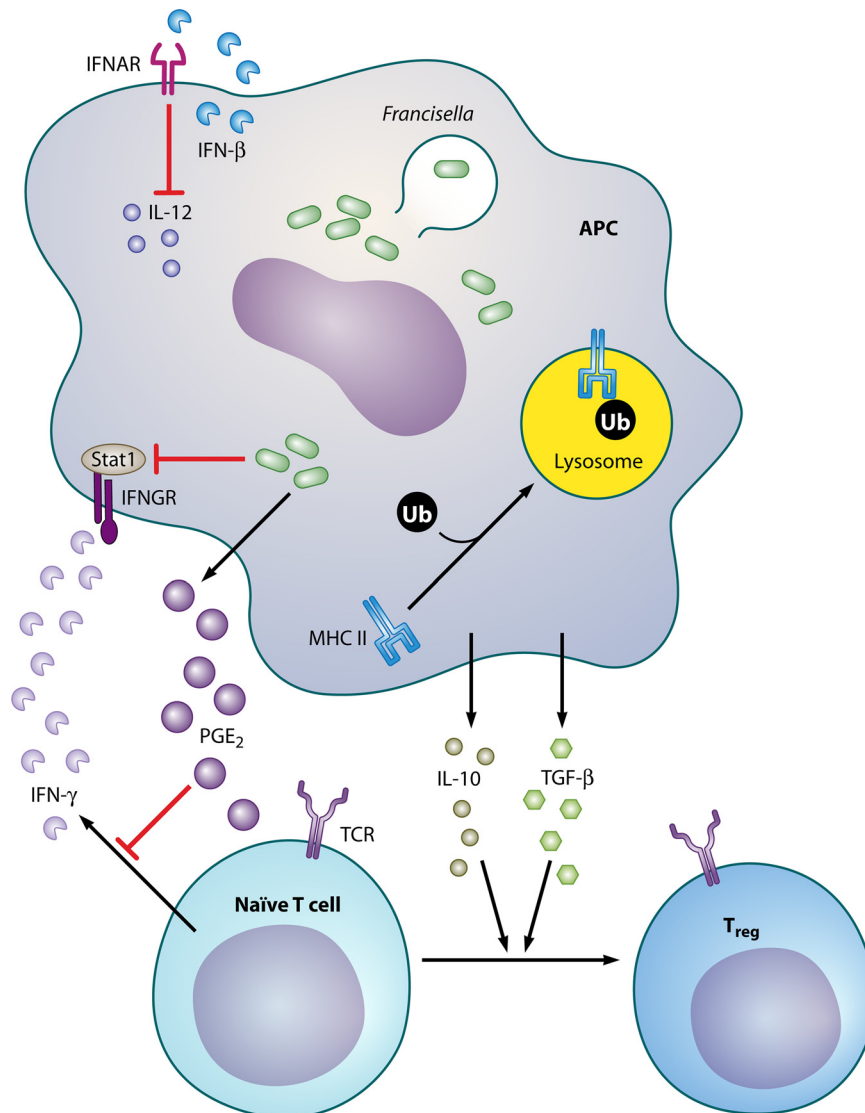
FIG 7 Subversion of adaptive immune responses by *Francisella*. *Francisella* infects antigen-presenting cells (APCs) and dampens their ability to produce proinflammatory cytokines. IL-12 is blocked via the induction of beta interferon (IFN-β). IFN-γ is produced by dendritic cells and T cells during *Francisella* infection, and it induces macrophages to kill the bacteria. *Francisella* blocks signal transduction through the IFN-γ receptor (IFNGR) by inhibiting Stat1 phosphorylation and through the induction of prostaglandin E2 (PGE$_2$), which inhibits IFN-γ production by T cells. *Francisella* also hampers T cell activation by inducing the degradation of major histocompatibility complex class II (MHC II) (indicated by its absence from the cell surface, tagging with ubiquitin [Ub], and trafficking toward the lysosome) and directing APCs to produce the anti-inflammatory cytokines TGF-β and IL-10, which promote the development of regulatory T cells (T$_{reg}$) that are able to suppress inflammation and cell-mediated immune responses.

activation during the initial stages of infection but also helps provide a suitable environment for replication in subsequent phases of infection by dampening adaptive responses such as the activation of CD8 T cells, which could kill infected host cells harboring *Francisella*.

CONCLUDING REMARKS

Francisella uses a vast arsenal of virulence mechanisms to subvert host defenses and promote intracellular replication. The main overarching strategy is to prevent the initiation of host inflammatory responses. Since the inflammatory response operates in a positive-feedback loop, it is powerful once started and very difficult, if not impossible, to reign in. If initiated, host defenses encountered

by the bacteria (extracellular, phagosomal, and cytosolic defenses) are heightened at each step of infection, taking a toll on replication levels. This is highlighted by comparing the intracellular fates of *Francisella* in unstimulated or IFN-γ-stimulated macrophages. While *Francisella* is able to grow rapidly in unstimulated macrophages, IFN-γ-stimulated macrophages are not permissive for replication and are instead able to kill the bacteria. Therefore, if inflammatory processes leading to IFN-γ production are initiated, this drastically alters the course of infection in favor of the host.

Since PAMPs activate inflammatory pathways during infection, a critical aspect of subverting the inflammatory response is preventing PAMP recognition by the host. *Francisella* achieves this in large part by devoting considerable resources to resisting

damage (capsule, LPS O antigen, lipid A modifications, efflux pumps, detoxifying ROS, etc.) induced by antimicrobials that would lead to the release of PAMPs. When PAMPs are inevitably liberated and recognized by the host at some level, *Francisella* suppresses the signaling of host PRRs, although the mechanisms by which this occurs are not defined and will be a critical area of future research. The importance of subverting early inflammatory responses can be seen when studying numerous mutant strains that lack virulence traits required in the first hours of infection (resistance to complement and phagosomal defenses, defects in phagosome escape, etc), which are often hyperinflammatory and severely attenuated for virulence.

Remaining immunologically "silent" allows the bacteria to reach the host cytosol, where they can replicate. However, an often overlooked virulence trait is the ability to acquire sufficient nutrients to facilitate rapid intracellular replication. *Francisella* has several strategies to acquire iron and synthesize tryptophan *de novo* to subvert specific host defense pathways. As mentioned, numerous metabolic genes have been identified in virulence screens and may lead to the elucidation of novel mechanisms by which the bacteria subvert "nutritional defenses." This is a subject of great interest not just for *Francisella* pathogenesis but also in the field of bacterial pathogenesis as a whole. As it is one of the most virulent bacterial pathogens known, continued research into how *Francisella* subverts innate and adaptive host responses will lead to novel discoveries that will enhance our understanding of host-pathogen interactions.

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